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Transfer of *Haemophilus influenzae* Chromosomal Genes by Cell-to-Cell Contact

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A low-frequency exchange of chromosomal markers was observed in matings of *Haemophilus influenzae*. Transfer did not appear to be due to classical transformation or to be plasmid mediated, and chromosomal gene transfer differed in several respects from plasmid transfer by conjugation

The transfer of chromosomal genes in *Haemophilus influenzae* by transformation has been well described (7, 10, 14), and the transfer of plasmid genes by a process that resembles conjugation has also recently been described for this species (16, 17). As part of an ongoing study of the mechanisms of plasmid-mediated antibiotic resistance transfer in *H. influenzae* (1, 2, 12), we undertook a series of experiments designed to demonstrate the direction of gene transfer under conditions of cell-to-cell contact and observed the transfer of chromosomal genes. Further studies reported here have led us to believe that non-plasmid-mediated chromosomal gene transfer occurs in this species by a mechanism other than classical transformation.

MATERIALS AND METHODS

All strains of *H. influenzae* used have been previously described. Strain Rd is a transformable derivative of the capsular type d strain isolated by Alexander and Leidy (3). The mutants *rec-1* and *rec-2* are recombination-deficient strains derived from strain Rd (9, 11). The *rec-1* Str^r(p2265) strain was selected from a transconjugant of *rec-1* and a strain containing the conjugative plasmid p2265 (1). Novobiocin and streptomycin markers conferred resistance to these antibiotics at 2.5 and 250 μg/ml, respectively.

Transformation was carried out with cells made competent in MIV medium (15) by using chromosomal DNA prepared by the method of Marmur (8) from a strain resistant to streptomycin.

Matings were carried out by using mid-log-phase cells at donor-to-recipient ratios of 1:1 either on membrane filters on hemoglobin agar as described previously (2), by cogrowth overnight on hemoglobin agar, or in supplemented brain-heart infusion broth at cell densities greater than 10^8. Cultures were left at 37°C for 2 h under these conditions unless otherwise noted.

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RESULTS

Chromosomal transfer under conditions of cell-to-cell contact in the absence of known plasmids. Although the initial observation of chromosomal gene transfer was made in matings involving strains containing plasmids, it seemed important to attempt to establish the phenomenon in strains not containing plasmids. The results of one such experiment are presented in Table 1. The strains used for this experiment have been repeatedly examined for plasmids by agarose gel electrophoresis of cleared lysates (12), and none has ever been found. For the first five pairs of strains, the frequency of doubly resistant cells obtained is low but significantly above the spontaneous mutation rates for these markers, which were measured as being well below 10^{-8} in the cultures of these strains used for the experiment.

Effect of *rec* mutations on chromosomal transfer. Table 1 showed that one member of the mating pair must have an intact recombination system for chromosomal gene transfer to occur. Forty doubly resistant strains arising from matings in which one of the mating pair was recombination deficient were tested for transforming ability and found to be recombination proficient.

Evidence that chromosomal transfer under conditions of cell-to-cell contact is different from plasmid transfer and from classical transformation. Earlier studies of conjugative transfer of antibiotic resistance plasmids in *Haemophilus* spp. have shown a requirement for close cell-to-cell contact (2, 16, 17). We looked at the transfer of plasmid and chromosomal markers under conditions of liquid mating and during cogrowth on a solid agar surface (Table 2). It is clear that the efficiency of transfer of a plasmid marker was markedly increased by efficient cell-to-cell contact during cogrowth on solid medium, but

TABLE 1. Transfer of chromosomal markers between strains of *H. influenzae* during filter matings on solid agar[a]

Strains and resistance markers	No. of Novr Str^r colonies on 20 plates[b]	No. of Str^r cells/ml[c]	No. of Novr cells/ml[c]	Frequency of doubly resistant Novr Str^r/Nov^r
Rd Str^r × Rd Nov^r	805	4.8×10^8	2.5×10^8	1.6×10^{-6}
Rd Str^r × *rec-1* Nov^r	224	3.0×10^8	5.4×10^7	2.1×10^{-6}
Rd Str^r × *rec-2* Nov^r	83	1.1×10^8	1.6×10^8	2.6×10^{-7}
Rd Nov^r × *rec-1* Str^r	5	2.0×10^6	2.9×10^8	8.6×10^{-7}
Rd Nov^r × *rec-2* Str^r	51	1.9×10^8	1.5×10^8	1.7×10^{-7}
rec-1 Str^r × *rec-1* Nov^r	0	4.3×10^7	4.1×10^8	$<1.2 \times 10^{-9}$
rec-1 Str^r × *rec-2* Nov^r	0	3.0×10^6	1.8×10^8	$<2.8 \times 10^{-9}$
rec-2 Str^r × *rec-1* Nov^r	0	7.2×10^8	2.1×10^8	$<2.4 \times 10^{-9}$
rec-2 Str^r × *rec-2* Nov^r	0	9.1×10^7	1.9×10^8	$<2.6 \times 10^{-9}$

[a] Str^r, Streptomycin resistant; Nov^r, novobiocin resistant.

[b] Cells were removed from the agar after overnight incubation and suspended in cold Eugonbroth (BBL Microbiology Systems, Cockeysville, Md.). A 0.1-ml portion was plated directly from the suspension 20 times on the two antibiotics.

[c] The suspensions were diluted appropriately and plated on the indicated antibiotic.

there was little, if any, effect on the transfer of chromosomal markers. Furthermore, the efficiency of chromosomal gene transfer was not affected by the presence of a plasmid in the donor strain (cf. Table 1). Such results would be consistent with the transfer of chromosomal genes by classical transformation on the release of DNA into the medium by a proportion of the population.

Several experiments were carried out to look for evidence of classical transformation as a mechanism of chromosomal gene transfer between mating cells. First, cells were made competent in MIV medium and assayed for transfer of plasmid and chromosomal genes (Table 2). The development of competence had little or no effect on the transfer of plasmid markers but improved the recovery of chromosomal markers by a little more than one order of magnitude. Second, the effect of DNase on the transfer of plasmid and chromosomal markers was assessed under similar conditions (Table 2). Again, DNase had no effect on the transfer of plasmid genes into noncompetent cells but reduced the transfer of chromosomal genes by less than one order of magnitude. Finally, transformation of *H. influenzae* requires the presence of a specific base sequence on the donor DNA for uptake (5, 13) and can be competitively inhibited by unmarked homologous DNA. The transfer of chromosomal markers was markedly inhibited by unmarked DNA when transfer was by transformation, but only minimal or no inhibition was seen when transfer was by conjugation under comparable conditions (Table 3). It should be noted that although DNase at 125 μg/ml abolished all transfer of chromosomal genes to noncompetent cells during transformation by free DNA, it had only a relatively small effect on the transfer of chromosomal genes during conjugation of noncompetent cells. Similar results with DNase were found with noncompetent cells in the experiment shown in Table 2. The inability of DNase to eliminate the transfer of chromo-

TABLE 2. Effect of various conditions related to transformation on the transfer of chromosomal and plasmid genes in *H. influenzae* during mating of Rd Novr and *rec-2* Str^r(p2265) cells[a]

Mating conditions	No. of Novr cells/ml	No. of Str^r cells/ml	No. of Str^r Novr cells/ml	No. of Tet^r Novr cells/ml	Frequency of Str^r Nov^r/Nov^r	Frequency of Tet^r Nov^r/Nov^r
Liquid medium						
Noncompetent cells	6.3×10^8	5.3×10^8	6.8×10^2	5.4×10^5	1.1×10^{-6}	8.6×10^{-4}
DNase (125 μg/ml) added	1.1×10^9	1.2×10^9	3.3×10^2	8.2×10^5	3.0×10^{-7}	7.5×10^{-4}
Competent cells	1.1×10^9	7.9×10^8	4.2×10^4	4.5×10^5	3.8×10^{-5}	4.1×10^{-4}
Solid medium						
Noncompetent cells	5.8×10^7	1.8×10^9	1.5×10^2	1.3×10^8	2.6×10^{-7}	2.2×10^{-1}
DNase (125 μg/ml) added	4.2×10^8	9.3×10^8	6.7×10^1	1.9×10^8	1.6×10^{-7}	4.5×10^{-1}
Competent cells	1.0×10^9	2.1×10^9	7.2×10^3	2.1×10^8	7.2×10^{-6}	2.1×10^{-1}

[a] Nov^r, Novobiocin resistant; Str^r, streptomycin resistant; Tet^r, tetracycline resistant.

TABLE 3. Effect of DNase and competition by unmarked DNA for transfer of chromosomal markers during conjugation and transformation in noncompetent cells

Transformation[a]			Conjugation[b]		
Final concn of competing DNA (μg/ml)	No. of streptomycin transformants/ml	Fraction of Str^r recipients	Final concn of competing DNA (μg/ml)	No. of doubly resistant transconjugants/ml	Fraction of doubly resistant transconjugants
56	2.8×10^2	1.7×10^{-7}	112	2.4×10^3	4.2×10^{-6}
5.6	8.5×10^2	5.1×10^{-7}	11.2	2.2×10^3	3.5×10^{-6}
0.56	1.7×10^3	1.0×10^{-6}	1.12	2.5×10^3	4.4×10^{-6}
0.056	1.6×10^3	9.0×10^{-7}	0.112	2.3×10^3	3.6×10^{-6}
0	1.6×10^3	1.0×10^{-6}	0	2.5×10^3	4.1×10^{-6}
0 + DNase (125 μg/ml)	0	$<7.0 \times 10^{-10}$	0 + DNase (125 μg/ml)	0.95×10^3	1.7×10^{-6}

[a] Rd strain transformed with 0.02 ug DNA carrying a streptomycin resistance marker in standing liquid culture. The total number of cells at the time of plating was 1.6×10^9/ml.
[b] Rd Nov^r × *rec-1* Str^r(p2265) mating in standing liquid culture. The total number of cells at the time of plating was 0.9×10^9/ml.

somal genes in these experiments is not the result of inactivation of the enzyme, since DNase incubated with cells for 1.5 h in standing liquid culture was still fully active against transforming DNA (1 μg/ml) (all transforming activity was eliminated in 15 min).

Lack of ordered transfer of chromosomal DNA. A time-of-entry experiment in which strain pairs were incubated on filters on hemoglobin agar, resuspended, and plated at various times during incubation at 37°C is shown in Table 4. One member of the pair was a *rec-1* derivative carrying four different antibiotic resistance markers. The recipient was an Rd strain resistant to 50 μg of rifampicin per ml. Because of our evidence that the Rec$^-$ strain is the donor, we assumed that all of the doubly resistant cells arose from transfer from *rec-1* to the Rd strain. The order of the markers on the genetic map is novobiocin, streptomycin, erythromycin, and nalidixic acid resistance, separated by about 290 megadaltons of DNA (J. W. Bendler, personal communication). The data in Table 4 showed no correlation between the map and the order in which the markers are acquired by the recipient cell. A similar conclusion was drawn from four other experiments of this type.

TABLE 4. Marker transfer during interrupted mating between *rec-1* cells resistant to erythromycin, nalidixic acid, novobiocin, and streptomycin and Rd cells resistant to rifampicin

Time on filter (min)	No. of cells/ml resistant to[a]:					
	Str (10^8)	Rif (10^8)	Str Rif (10^2)	Nov Rif (10^2)	Ery Rif (10^2)	Nal Rif (10^2)
30	1.7	4.3	2.2	1.2	1.0	2.8
50	2.6	6.3	5.0	9.2	6.0	9.8
70	4.2	8.7	46	41	33	37
120	7.7	17	92	114	83	104

[a] Str, streptomycin; Rif, rifampicin; Nov, novobiocin; Ery, erythromycin; Nal, nalidixic acid.

Possibility of cell fusion. The data did not eliminate the possibility of a small amount of cell fusion rather than chromosomal gene transfer to explain the doubly resistant cells. If there were cell fusions resulting from cogrowth of a multiply marked strain and a singly marked strain, then any cells containing one marker from each participant should also contain other markers. In an experiment similar to that shown in Table 4, this did not appear to be the case. The frequency of cells resistant to streptomycin, nalidixic acid, and rifampicin together was several orders of magnitude lower than the frequency of doubly resistant cells. Similar frequencies of triply resistant cells were obtained in other experiments and always were considerably lower than the frequencies of doubly resistant cells. We conclude that cell fusion was not responsible for our observations of chromosomal gene transfer.

DISCUSSION

We have presented evidence that chromosomal gene transfer occurs in strains of *H. influenzae* under mating conditions which allow the conjugal transfer of plasmid genes but does not require the presence of plasmids in either the donor or the recipient strain. We have also presented evidence that this type of chromosomal gene transfer is different from classical transformation, although many of the features of cell-to-cell transfer do indeed resemble those of classical transformation.

Cell-to-cell transfer shares with transformation the dependence on *rec*$^+$ genes, and increased transfer is seen with the development of competence. Since chromosomal gene transfer,

no matter what the mechanism of DNA entry, involves recombination with the chromosome of the recipient, we would expect the requirement for rec^{+} gene products. Competence does increase the cell-to-cell transfer of chromosomal genes, although the increase is several orders of magnitude smaller than the increase in transformation observed when cells are made competent (6). Again, we would expect an effect of competence on cell-to-cell chromosomal gene transfer because recombination with the recipient chromosome is involved. In competent cells there is a special configuration of chromosomal DNA favorable for pairing with transforming DNA (7). The competent *rec-2* mutant, whose DNA does not assume this configuration, takes up DNA from the medium as well as does the wild-type strain but is transformed at a level of 10^{-7} times that of the wild type. Furthermore, recombination of *H. influenzae* phage in competent cells is about one order of magnitude higher than that in noncompetent cells (4). These data suggest that the enzymatic machinery for recombination is more active in competent than in noncompetent cells.

Cell-to-cell chromosomal transfer, like classical transformation, was decreased by DNase. However, when the same amount of DNase was present under the same conditions of dense cell suspension, transformation was totally abolished, but there was only a relatively small effect on cell-to-cell transfer. Thus, there was a large quantitative difference between the effect of competence and DNase on transformation and the effect on cell-to-cell gene transfer. Furthermore, transformation was affected by competing DNA and cell-to-cell transfer was not, under similar conditions of dense cell suspension.

It also seems unlikely that there could be sufficient release of DNA into the medium to account for the observed chromosomal gene transfer. Each *H. influenzae* cell contains about 10^{-15} g of DNA (R. M. Herriott, personal communication), and if all of the *rec-1* cells of the experiment shown in Table 3 had lysed, the amount of DNA released to the medium would have been around 0.2 μg. Thus, the amount of transforming DNA added to the wild-type (Rd) cells for transformation in the experiment shown in Table 3 was equivalent to the lysis of around 10% of the donor cells. Since *H. influenzae* cells, even when they have been inactivated by most agents, do not lyse (Setlow, unpublished observations), we consider that the doubly resistant cells resulting from cogrowth of the strains of Table 3 did not arise from cell lysis followed by transformation.

Not only did cell-to-cell chromosomal gene transfer appear to be different from transformation, but it also differed from plasmid transfer. Whereas the establishment in a cell of a chromosomal gene acquired from another cell required wild-type *rec-1* and *rec-2* genes (Table 1), the establishment of a plasmid such as p2265 after conjugation is independent of these genes and thus is presumed not to involve recombination (1; Albritton, Setlow, and Slaney, unpublished data).

Chromosomal gene transfer in cell-to-cell matings differed from plasmid gene transfer in three other respects. Neither competence nor DNase affected plasmid transfer, although both affected chromosomal transfer. Transfer of plasmids was more than two orders of magnitude more probable on solid agar than in liquid culture, whereas chromosomal transfer was similar under the two conditions. Therefore, we propose that cell-to-cell chromosomal transfer takes place by a different mechanism than that of plasmid transfer. Since it is apparently random with respect to the genetic map, and since it takes place in the absence of plasmids, the mechanism of cell-to-cell chromosomal transfer is probably also different from conjugal transfer in *Escherichia coli* Hfr strains (although our time-of-entry data did not exclude the possibility that there was ordered transfer starting at different sites on the genome in different cells). We further propose that the partial DNase sensitivity of cell-to-cell chromosomal transfer is the result of the particular type of contact required for the exchange of chromosomal DNA.

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